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Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1–5 against nervous necrosis virus in medaka

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ABSTRACT

The nervous necrosis virus (NNV)-medaka infection model was used in this study for analysis of NNV infection and treatment of NNV with the antimicrobial peptides (AMPs) of epinecidin-1 and hepcidin 1–5 at the organismal level. Our results showed that co-treatment of AMPs with the virus was effective in promoting a significant increase in medaka survival. Re-challenge with the virus also showed high survival suggesting that these two AMPs enhanced fish survival. However, pretreatment or post-treatment with AMPs showed that both of these AMPs increased medaka survival and suggested that AMPs can be used as drugs to rescue infected medaka. The data presented here indicate that epinecidin-1 and hepcidin 1–5 have *in vivo* antivirus activity against the NNV, and hepcidin 1–5 functions like a lytic peptide after an *in vitro* assay. Infection after pretreatment, co-treatment, and post-treatment with epinecidin-1 or hepcidin 1–5 was verified by RT-PCR which showed both peptides can downregulate NNV and interferon gene expressions. In addition, our results suggest that epinecidin-1 or hepcidin 1–5 may prove to be an effective chemotherapeutic agent for aquaculture in the future.

1. Introduction

Viral infections are serious problems that disrupt aquaculture fishery production and cause substantial economic losses each year. One important pathogen, a nodavirus, is responsible for viral encephalopathy and retinopathy (VER) disease which induces high mortalities in infected fish. Nodaviruses are small (25–30 nm in diameter), spherical, non-enveloped viruses with a genome that is composed of two single-stranded, positive-sense RNA molecules. RNA1 (3.1 kb) encodes RNA-dependent RNA polymerase, which belongs to the large genomic segment, and RNA2 (1.4 kb) encodes the coat protein that belongs to the smaller genomic segment [16,28,30]. In addition, the family Nodaviridae contains two genera, one of which contains betanodaviruses which usually infect fish, while the other contains alphanodaviruses which almost always infect insects [27]. According to the RNA2 sequences, phylogenetic analyses classified them into four genotypes, designated barfin flounder nervous necrosis virus (BFNNV), tiger puffer NNV (TPNNV), striped jack NNV (SJNNV), and red spotted grouper NNV (RGNNV) [30]. Most NNVs infect nervous tissues and other tissues depending on the fish species and age; for example, NNV was detected in the epithelium of hyperplasic skin of striped jack larvae [29] and Atlantic halibut larvae [15]. Infected fish commonly display neurological disorders, which are often associated with strong vacuolization of the central nervous system (CNS) and retina which causes the fish to swim in a circular pattern. However, the pathogenesis of NNV infection in which acute infection in larvae and juvenile stages caused mass mortality while persistently infecting adult remains poorly understood.

Fish diseases are the greatest problem facing aquaculture and damaging its profitability, and they have caused direct losses in excess of several million US dollars to the aquaculture industry worldwide in recent years. Although fish diseases can be prevented and controlled by vaccinations [24], there is still no truly effective vaccine or miracle drug which can treat a variety of fish diseases caused by betanodaviruses. At present, there are several types of NNV vaccines which have been developed including recombinant proteins, synthetic peptides, inactivated virions, DNA vaccines, and virus-like particles by an intramuscular or intraperitoneal injection or by bath immunization [10,19,40]. However, in most cases mentioned above, we found it was very difficult to inject the vaccine into larvae and juvenile fish because NNV-infected fish are usually in the early development stages. Therefore, the development of new drugs to prevent or treat NNV infection is an urgent step for rescuing infected fish.

The factors that contribute to NNV-induced disease are largely unknown. A part of a subset of RGNNV-infected fish develop extensive cellular vacuolation and neuronal degeneration by necrosis in the CNS, which raises the question of what unique factors are present in these situations causing pathogenesis to ensue? The factors that contribute to NNV-induced neuronal degeneration likely comprise multiple conditions that involve the virus,

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fish, and environment. Molecular biological studies suggested that there is a significant cofactor involved in induction of apoptosis by the RGNNV TN1 strain followed by secondary necrosis in fish cells leading to NNV-induced host secondary necrosis through a caspase-independent pathway [37]. The rise in NNV-infected fish diagnoses has directly spurred the search for more-potent antiviral drugs to interrupt signal transduction pathways. Currently, enhancing a fish's immune ability and reducing viral binding to host cells are treatments effectively used to decrease viral replication in infected fish, suggesting that a functional immune system and development of new drugs (or new adjuvants) are critical for controlling NNV replication and pathology [25]. Some work focused on determining which components of betanodaviruses modulate cell death in the early replication cycle in fish cells or if betanodavirus non-structural protein B2 can induce mitochondrion-mediated cell death in fish cells [34]. A robust interferon (IFN) response in NNV-infected fish was correlated with decreased NNV replication, whereas a weak viral RNA amount was detected after differential IFN treatment [26]. Together those studies indicated that virally induced host cell-mediated response immunity is very important in controlling NNV replication and spread in aquatic animal hosts. Although there are a few reports describing the mechanism of betanodavirus infection [5,26], virtually nothing is known about the role of the fish innate immune system in NNV infection.

Recent reports have drawn attention to the antiviral activity of antimicrobial peptides (AMPs) that were first defined by their antibacterial properties [14,39]. For example, alpha-defensins were shown to inhibit HIV-1 infection *in vitro* through binding to HIV-1 gp120 and CD4 [36]. LL-37 (cathelicidin in humans) binding to the formyl peptide receptor like-1 receptor induces downregulation of HIV-1 chemokine receptors and reduces susceptibility to HIV-1 infection *in vitro* [2,11]. With these data in mind, it was natural to hypothesize that AMPs from fish, like mammalian AMPs, would inhibit NNV infection through their antiviral activities. The aim of this study was to examine if fish AMPs such as epinecidin-1 and hepcidin 1–5 block NNV infection in medaka (*Oryzias latipes*). Epinecidin-1 and hepcidin 1–5 are small, cationic peptides that show broad activities against fungi and bacteria depending on the individual antimicrobial spectrum [18,31,32]. Interestingly, injection of poly I:poly C induced the expression of epinecidin-1 in grouper (*Epinephelus coioides*) and hepcidin 1–5 in tilapia (*Oreochromis mossambicus*) [18,31]. In this study, we reveal that two peptides, epinecidin-1 and hepcidin 1–5, significantly inhibited NNV infection with AMP pretreatment, post-treatment, and co-treatment in medaka which suggests that both peptides may be good candidates for development as drugs to cure virally infected fish or as immune enhancers.

2. Materials and methods

2.1. Fish

Adult medaka (weighing 1.43 ± 0.25 mg) was purchased from the Taikong Company (Taipei, Taiwan), were maintained at 27 °C throughout the experimental period, and were fed commercial fodder daily. Prior to the experiments, the medaka was acclimated to laboratory conditions for 21 days.

2.2. Peptides and viruses

The tilapia hepcidin TH1-5 sequence was GIKCRFCCGCCTPGICGVCCRF [18], and the epinecidin-1 sequence was GFIFHIIKGLFHAGKMIHGLV [32]. Both peptides were synthesized with an amidated C-terminus by GL Biochem (Shanghai, China) at >95% purity. The synthetic peptides were reconstituted in phosphate-buffered saline (PBS; pH 7.4) for the experiments.

The virus was a gift from Dr. Shau-Chi Chi's laboratory (Institute of Zoology, National Taiwan University, Taipei, Taiwan). Briefly, the viral purification procedures followed a previous publication [6], and the virus was stored in PBS for further experiments.

2.3. Effects of epinecidin-1 and hepcidin 1–5 on viral challenge in medaka

A model of fulminating virus (GNNV 9508 strain) lethality [6,8,19] was used to characterize the efficacy of the synthesized AMPs of epinecidin-1 and hepcidin 1–5 for protecting medaka against viral infection by injection. The first trial of every group used 33 medaka and there are 6 groups in first trial: (1) group 1 was only injected with 10 μl virus (at a titer of 10^8 TCID50/ml) in each fish; (2) in group 2, the virus (at a titer of 10^8 TCID50/ml; 10^6 TCID50/fish) and epinecidin-1 (100 μg/ml; 1 μg/fish) or hepcidin 1–5 (1000 μg/ml; 10 μg/fish) were mixed for 10 min and then injected; (3) in group 3, virus (at a titer of 10^8 TCID50/ml; 10^6 TCID50/fish) and epinecidin-1 (50 μg/ml; 0.5 μg/fish) or hepcidin 1–5 (100 μg/ml; 1 μg/fish) were mixed for 10 min and then injected; (4) in group 4, virus (at a titer of 10^8 TCID50/ml; 10^6 TCID50/fish) and epinecidin-1 (10 μg/ml; 0.1 μg/fish) or hepcidin 1–5 (10 μg/ml; 0.1 μg/fish) were mixed for 10 min and then injected; (5) in group 5, virus (at a titer of 10^8 TCID50/ml; 10^6 TCID50/fish) and epinecidin-1 (5 μg/ml; 0.05 μg/fish) or hepcidin 1–5 (1 μg/ml; 0.01 μg/fish) were mixed for 10 min and then injected together; and (6) group 6 was only injected with PBS.

For the second trial, each group contained 33 fish, and each fish was injected with 10 μl of an AMP of either epinecidin-1 (100 μg/ml) or hepcidin 1–5 (1000 μg/ml) and after 2, 4, or 8 h, 10 μl of RGNNV (10^8 TCID50/ml) was injected; the mortality rate was recorded every 24 h. However, the second trial also included another two groups: one was only injected with PBS and the other was only injected with 10 μl virus (at a titer of 10^8 TCID50/ml) in each fish.

For the third trial, each group contained 33 medaka, and each fish was injected with 10 μl virus (at a titer of 10^8 TCID50/ml) and after 8, 24, 48, or 72 h, epinecidin-1 (100 μg/ml) or hepcidin 1–5 (1000 μg/ml) was injected; the mortality rate was recorded every 24 h. For the fourth trial, each group contained different numbers of fish that had survived after the 31-day experimental period of trial 1 from the first day of co-injection of AMP and virus mixture. Each fish was injected with the virus (at a titer of 10^8 TCID50/ml; 10^6 TCID50/fish) again, and the mortality rate was recorded every 24 h. The PBS and NNV groups used new fish which were of the same age from the first trial, but had received no previous treatment. All administrations were given through the anal canal (cloaca). Fish were maintained in 5-l tanks at approximately 27 °C. Mortality was recorded daily, and fish were fed a commercial diet (Golden Prawn Enterprise, Kaohsiung, Taiwan) by hand twice a day. Mortality values are presented as percentages (%). All experiments were repeated with three independent tests. Values are presented as the mean ± S.E. of n experiments, where n represents the number of separate experiments for each RNA source. The significance was calculated by the SPSS statistical program and was set to $p < 0.05$.

2.4. Electron microscopy

The procedures of transmission electron microscopy (TEM) followed the protocols of previous publications with no modifications [31,32]. Briefly, the virus was mixed with a freshly prepared AMP solution in PBS to a final concentration of 100 μg/ml epinecidin-1, 500 μg/ml hepcidin 1–5, and 1000 μg/ml hepcidin 1–5 for 30 min. The control group consisted of only a viral suspension in PBS. After